IN THE SEQUENCE LISTING:

Please cancel from the application Original Sequence Listing pages 1-2 and substitute therefor the attached Replacement Sequence Listing pages 1-3.

REMARKS

This Response to Notice to Comply brings the patent application into compliance with the Sequence Listing Disclosure requirement of the United States Patent and Trademark Office. Enclosed herewith are: (1) a paper copy of the Replacement Sequence Listing, (2) and a computer readable version of the Replacement Sequence Listing. The Response to Notice to Comply directs entry of the paper copy of the Sequence listing into the application. In view of the foregoing, the application is believed to fully comply with the Sequence Listing Disclosure requirements.

VERIFICATION UNDER 37 C.F.R. § 1.821(f)&(g)

All of the sequences in the attached Sequence Listing were included in the application as filed. Pursuant to 37 C.F.R. § 1.821 (g), no new matter is being added herewith. As required under 37 C.F.R. § 1.821 (f), I hereby verify that the data on the enclosed disk and the paper copies of the Sequence Listing are identical.

CONCLUSION

No fees are believed due; however, should any fees be required, please charge them to Deposit Account No. 11-1410. Should there be any questions concerning this application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 21 June 2001

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VERSION WITH MARKINGS TO SHOW CHANGES

The paragraph beginning at page 4, line 11 has been amended as follows:

PAR1 and PAR2, but not PAR3 (Ishihara *et al.*, 1997) can also be activated by short synthetic peptide sequences corresponding to those of the tethered ligands. For PAR1, this tethered ligand is **[SFLLRN-NH_2 (single amino acid sequence[<400>1]]** (SEQ ID NO:1), which is also known as TRAP (thrombin receptor-activating peptide). The tethered ligand sequence for mouse PAR2 is **[SLIGRL-NH_2 [<400>2]]** (SEQ ID NO:2), and is referred to herein as PAR2 activating peptide (PAR2-AP). Therefore, these peptides can be used to mimic enzyme mediated PAR activation and to study the effects of PAR activation.

The paragraph beginning at page 7, line 17 has been amended as follows:

The present invention is predicated in part on the identification of airway epithelial PARs which modulate bronchodilation and inflammation. More particularly, the inventors have identified PARs in airway epithelium which, upon activation, simulate, induce or otherwise facilitate inhibition of bronchoconstriction and/or inflammation in humans **[an]** and animals.

The paragraph beginning at page 13, line 18 has been amended as follows:

The agent may be a nucleotide sequence, low molecular weight compound, or a derivative, part, fragment, analogue, mimetic, mimotope or chemical equivalent of all or a portion of PAR2. In particular, the agent may be a peptide having similar biological activity to **[SFLLRN-NH_2 [<400>1]]** (SEQ ID NO:1) and/or **[SLIGRL-NH_2 [<400>2]]** (SEQ ID NO:2) and/or **[SLIGKV-NH_2 [<400>3]]** (SEQ ID NO:3) (see Blackhart *et al.*, 1996). Gene therapy may also be employed such as using cationic liposomes for gene vector transfer.

The paragraph beginning at page 14, line 10 has been amended as follows:

Composition suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparations of sterile injectable solutions. They are generally stable under the condition of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, **[polyoil]** polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The preventions of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens,

chlorobutanol, phenol, sorbic acid, thrimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

The paragraph beginning at page 24, line 8 has been amended as follows:

Figure 1 is a schematic representation of the PAR2 receptor. The black loops depict the membrane-spanning regions in a theoretical cell. The receptor is activated by trypsin (or by other trypsin-like proteases, e.g. tryptase) by cleavage of the arginine^{34}-serine^{35} peptide bond amino-terminally to the arginine^{34} in the extracellular N-terminal domain. The next approximately six amino acids of the new N-terminal (called the tethered ligand sequence, solid box) now 'flip' on to another, undefined region of the remaining receptor to initiate intracellular G-protein (G) coupling and signaling, shown here as "responses". The putative tethered ligand binding region ("R") of the receptor can also be directly activated by exogenous addition of a synthetic peptide identical or homologues to the tethered ligand sequence **[SLIGRL-NH_2 (single letter amino acid code)]** (SEQ ID NO:2) designating the mouse PAR2 activating sequence. The similar but genetically distinct PAR1, or thrombin receptor, is enzymatically activated by thrombin by cleaving a arginine^{41}-serine^{42} bond and the synthetic tethered ligand sequence **[SFLLRN-NH_2]** (SEQ ID NO:1) designating the human PAR2 activating sequence.

The paragraph beginning at page 24, line 24 has been amended as follows:

Figure 2 is a chart recording showing changes in isometric force in a ring of mouse bronchus contracted to approximately 70% maximal force (F_{max}) with carbachol. The characteristic spontaneous fluctuations in active force fell markedly, then recovered at two points. After the second fall and recovery, extra carbachol was added to increase the level of active force above 70% F_{max}. A high, single concentration of the PAR2 activating peptide, PAR2-AP,**[(SFLLRN-NH_2)]** (SEQ ID NO:2) then induced a large relaxation.

The paragraph beginning at page 25, line 8 has been amended as follows:

Figure 4 is a chart recording showing changes in isometric force in a ring of mouse bronchus contracted to 40%-50% F_{max} with carbachol, and the effects of the PAR2 activating peptide, PAR2-AP **[(SLIGRL-NH_2)]** (SEQ ID NO:2) and trypsin in the absence and presence of nifedipine (0.3 μM).

The paragraph beginning at page 25, line 13 has been amended as follows:

Figure 5 shows the effect of removal of the epithelium on relaxation to the PAR2 activating peptide, PAR2-AP **[(SLIGRL-NH_2)]** (SEQ ID NO:2) in rings of the guinea pig

isolated bronchus. (A) Chart recordings of changes in isometric force in two rings contracted to 60%-70% F_{max} with carbachol (-logM) after which PAR2-AP was added (-logM). (B) Group data from six experiments described in (A). Responses are expressed as percentages of the contraction to carbachol and values are mean ±SEM. Positive values represent contractions.

The paragraph beginning at page 25, line 21 has been amended as follows:

Figure 6 is a chart recording demonstrating the obligatory role of the epithelium in mediating relaxation to the PAR2 activating peptide, PAR2-AP **[(SLIGRL-NH_2-logM)]** (SEQ ID NO:2, -logM), in isolated spiral strip preparations of the guinea pig bronchus. Strips were contracted to approximately 25% F_{max} with carbachol.

The paragraph beginning at page 26, line 1 has been amended as follows:

Figure 7 shows chart recordings depicting both the technique used to record relaxation in isolated mouse bronchial ring preparation, and the efficacy of the PAR2 activating peptide, PAR2-AP **[(SLIGRL-NH_2)]** (SEQ ID NO:2) and thrombin receptor activating peptide, TRAP **[(SFLLRN-NH_2)]** (SEQ ID NO:1) as broncho-relaxant agents. The time calibration bar represents 40 min, 12 min and 4 min during the F_{max} contraction, the 40% F_{max} contraction with carbachol and the additions of both peptides, respectively.

The paragraph beginning at page 26, line 9 has been amended as follows:

Figure 8 depicts the sensitivity and maximal relaxation to (A) **[SLIGRL-NH_2]** (SEQ ID NO:2) (PAR2-AP), (B) **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP), (C) trypsin and (D) thrombin in isolated mouse bronchial rings with epithelium and the effect of potential inhibitors of these responses. All responses are expressed as percentage relaxation of the initial levels of active force induced by carbachol (30%-60% F_{max}). Values are mean ±SEM from 6-9 experiments and positive values represent contractions. Drugs used were L-NOARG (100 μM), a nitric oxide (NO) synthase inhibitor; HbO (oxyhaemoglobin, 20 μM), a NO scavenger, and Indo (indomethacin, 3 μM) or aspirin (100 μM), both of which are cyclooxygenase inhibitors which prevent the synthesis of prostaglandin.

The paragraph beginning at page 28, line 20 has been amended as follows:

Figure 14 depicts chart recordings showing the relaxation to the PAR1-activating peptide **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP), but not the PAR2-activating peptide, **[SLIGRL-NH_2]** (SEQ ID NO:2) (PAR2-AP), in an isolated strip of epithelium-containing pig tracheal smooth muscle. **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP) caused a slow relaxation to near maximum to

that of isoprenaline, which showed a similar slow time course. The tissue was contracted to approximately 30% of its maximum contraction to acetylcholine (F_{max}) with carbachol. During the break in the trace (20 min), the tissue also recovered its 30% F_{max} level of active force spontaneously without washout.

The paragraph beginning at page 29, line 5 has been amended as follows:

Figure 15 depicts chart recordings showing relaxation to (A) PAR2 activating peptide (PAR2-AP) or **[SLIGRL-NH_2]** (SEQ ID NO:2) and (B) trypsin in two isolated ring preparations of the rat bronchi with intact epithelium. In each case, the tissue was contracted to 50%-70% of their maximum contraction (F_{max}) to acetylcholine (30 μM). R_{max} represents the maximum relaxation to isoprenaline.

The paragraph beginning at page 29, line 11 has been amended as follows:

Figure 16 depicts chart recordings showing relaxation to the PAR1 activating peptide **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP) in a single preparation of the guinea pig isolated taenia coli, which initially contracted repeatedly with histamine (HIST; 1 μM) to stable, submaximal levels of active force. At the breaks in the trace, the preparation was washed thoroughly and left to recover for approximately 30 min prior to the next contraction with histamine. (A), control; (B), after treatment with propranolol (1 μM) and prazosin (1 μM) to block any relaxant adrenoreceptora; (C), as for (B) except the NO synthase inhibitor, L-NOARG (100 μM), was added as well; (D) as for (C) except the small conductance, Ca^{2+}-activated K^+ channel (SK) inhibitor, apamin (0.1 μM), was added as well.

The paragraph beginning at page 29, line 22 has been amended as follows:

Figure 17 is a chart recording showing relaxation to the PAR2 activating peptide (PAR2-AP or **[SLIGRL-NH_2]** (SEQ ID NO:2)) and the PAR1 activating peptide **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP) in an isolated strip of rat gastric fundus in which the mucosa was left intact. The tissue was contracted to approximately 50% of its maximum contraction to KCl (50 mM) with acetylcholine (Ach). Isoprenaline was added to obtain maximum relaxation.

The paragraph beginning at page 30, line 5 has been amended as follows:

Figure 18 is a chart recording showing the relaxation to the PAR1 activating peptide ([**SFLLRN-NH_2**] (SEQ ID NO:1) or TRAP) [and to a smaller extent, PAR2-AP or **[SLIGRL-NH_2]** (SEQ ID NO:2)] in an isolated strip of longitudinal muscle of the human distal colon. The preparation was contracted to a stable level of active force with repeated additions of substance P

(SP, w = wash). The breaks in the trace represent 10-15 min. Apamin was left in contact with the preparation for more than 30 min.

The paragraph beginning at page 31, line 4 has been amended as follows:

Figure 21 shows the response to PAR1 and PAR2 activating peptides in human isolated coronary artery ring segments contracted to approximately 50% of their maximum contraction in response to 125 mM ($KPSS_{max}$) with U46619. Cumulative concentration-response curves were generated to the human PAR1 activating peptide, **[SFLLRN-NH_2]** (SEQ ID NO:1), in endothelium-intact (o, n=10 from 5 patients) and denuded (•, n=5 from 5 patients) preparations and to the human PAR2 activating peptide, **[SLIGKV-NH_2]** (SEQ ID NO:3), in endothelium-intact tissues (Π, n=5 from 2 patients). Data are expressed as mean ±SEM.

The paragraph beginning at page 31, line 13 has been amended as follows:

Figure 22 shows digitized traces of original chart recording showing the effect of desensitization to thrombin (a) and trypsin (b) on relaxation to the thrombin receptor peptide ligand, **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP), in separate rings of human coronary artery contracted to ~50% F_{max} with 3 nM (a) and 4 nM (b) final concentration of U46619. SP=substance P; ISO=isoprenaline; Throm=thrombin; Tryp=trypsin (units of both enzymes given as U/ml). The time calibration bar represents 20 min prior to the arrow. Tissues were incubated for at least 2 h with maximum concentrations of (a) thrombin and (b) trypsin, and then washed prior to contraction with U46619.

The paragraph beginning at page 31, line 23 has been amended as follows:

Figure 23 shows the effect of thrombin desensitization on response to the PAR1 activating peptide in human isolated coronary artery. Ring segments contracted to approximately 50% of their maximum contraction in response to 125 mM ($KPSS_{max}$) with U46619 were either untreated (o) or desensitized to both thrombin and trypsin (•) before cumulative concentration-response curves to the human PAR1 activating peptide, **[SFLLRN-NH_2]** (SEQ ID NO:1), were generated. Data are expressed as mean ±SEM (n=8, from 4 patients).

The paragraph beginning at page 32, line 22 has been amended as follows:

Figure 26 shows the immunohistochemical localization of PAR2 in mouse bronchi, and demonstrates that PAR2 and PAR1 mediates epithelium-dependent relaxation in isolated rings of this tissue.

(a) Confocal photomicrograph showing PAR2 immunofluorescence to discrete epithelial cells (arrow) as well as smooth muscle cells (m) and fibroblasts (arrow head). In some epithelial cells, the fluorescence appeared concentrated within areas of the cytoplasm. Pre-absorption with the peptide sequence used to raise the mouse PAR2 antibody quenched the epithelial, smooth muscle and fibroblast fluorescence. The scale bar represents 10 μm.

(b) An original, digitized chart recording of changes in isometric force in a single ring of mouse left bronchus with intact epithelium. The tissue was contracted to approximately 40% F_{max} to acetylcholine (Ach; 30 μM) with cumulative, titrated concentrations of carbachol. Note the change of gain, and that the force recovered spontaneously over the 15 minute break in the trace after maximum relaxation to **[SLIGRL-NH_2]** (SEQ ID NO:2).

(c) Removal of the epithelium with 0.1% v/v Triton X-100 abolished relaxation to **[SLIGRL-NH_2]** (SEQ ID NO:2) and **[SFLLRN-NH_2]** (SEQ ID NO:1) whereas the tissue could still sensitively relax to PGE_2.

(d) Light photomicrographs of cross sections of mouse bronchi, showing that the 0.1% Triton X-100 perfusion technique removed the vast majority of columnar epithelial cells (arrows) with no microscopic evidence of damage to the underlying smooth muscle (m). Scale bar represents 30 μm.

The paragraph beginning at page 34, line 3 has been amended as follows:

Figure 27 shows the mechanisms of PAR-mediated bronchodilation.

(a) epithelium-; and

(b) cyclooxygenase-dependent relaxations of mouse isolated bronchi to the PAR2 and PAR1 synthetic peptide ligands **[SLIGRL-NH_2]** (SEQ ID NO:2) and **[SFLLRN-NH_2]** (SEQ ID NO:1), respectively.

(c) Relaxations to trypsin and thrombin in epithelium-intact preparation were **[similary]** similarly abolished by cyclooxygenase inhibition. Group data from similar experiments to that described in Figure 26, except that tissues were treated with indomethacin (3 μM) and aspirin (100 μM) to block cyclooxygenase activity. All relaxation and contraction are expressed as percentages of the initial force to carbachol (40% F_{max}) regardless of treatment. Values on the graphs are

mean ± s.e. mean from 5-9 experiments, except aspirin (n=3). *P < 0.01. Note that the NO inhibitors had no effect on the relaxations to PAR1- and PAR2-activating peptides.

The paragraph beginning at page 34, line 22 has been amended as follows:

Figure 28 shows that the PAR1- and PAR2-activating peptides **[SFLLRN-NH_2]** (SEQ ID NO:1) and **[SLIGRL-NH_2]** (SEQ ID NO:2) respectively, act at separate receptors to cause bronchial relaxation. **[Desensitisation]** Desensitization to trypsin (Π) but not to thrombin (▲) abolished the responses to the PAR peptide, **[SLIGRL-NH_2]** (SEQ ID NO:2) (a), whereas relaxation to the PAR1 peptide, **[SFLLRN-NH_2]** (SEQ ID NO:1), was markedly inhibited following desensitization to both enzymes (b). In both cases, (•) represents control responses. Values on the graphs are mean ±s.e. mean from 5-6 experiments.

The paragraph beginning at page 35, line 6 has been amended as follows:

Figure 29 are graphical representations demonstrating that the PAR2 activating peptide **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) causes inhibition of bronchoconstriction *in vivo*. Original chart recordings (a, b) and grouped data (c, d) showing the effect of a 30 sec exposure to an aerosol of a 0.1 mg/ml solution of **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) on 5-HT (3 nmol/kg i.v.)-induced changes in airway resistance (R_L; a, c) and dynamic compliance (C_{dyn}; b, d) in the anaesthetized rat. Not shown is the complete inhibition of bronchoconstriction to 5-HT lasting at least 45 min occurred when **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) was used at 1 mg/ml. Values are mean ±s.e. from n=3 experiments.

The paragraph beginning at page 35, line 16 has been amended as follows:

Figure 30 is a graphical representation showing digitized traces of original chart recordings of increases in Isc to ATP, the PAR2 synthetic ligand; **[SLIGRL-NH_2]** (SEQ ID NO:2) and PGE2 in mouse trachea mounted in an Ussing Chamber. All drugs were applied on the apical surface.

The paragraph beginning at page 35, line 21 has been amended as follows:

Figure 31 is a graphical representation showing concentration-dependent increase in short circuit current (Isc) in mouse trachea mounted in the Ussing Chamber (see methods). Cumulative concentration of **[SLIGRL-NH_2]** (SEQ ID NO:2) (Π), **[SFLLRN-NH_2]** (SEQ ID NO:1) (▲), **[GYPGKF-NH_2]** (SEQ ID NO:6) (▼), PGE_2, (♦), ATP (•) and UTP (□) were added to the luminal bath. Values on the graphs are mean ±SEM from 5-6 experiments.

The paragraph beginning at page 36, line 3 has been amended as follows:

Figure 32 is a graphical representation showing the effect of CFTR and dependent Cl^{-Ca2+} channel inhibition by glibenclamide (100 μM: G) and DIDS (4, 4'-diisothiocyanostilbene 2, 2'-sulfonic acid, 100 μM: D) respectively on (a) **[SLIGRL-NH_2]** (SEQ ID NO:2) (30 μM), (b) ATP (10 μM) and (c) PGE_2 (10 μM). Digitized traces of original chart recordings for responses in mouse trachea mounted on an Ussing Chamber are shown. All drugs were applied on the apical surface.

The paragraph beginning at page 36, line 10 has been amended as follows:

Figure 33 is a graphical representation showing a mechanism of chloride secretion component of I_{sc}: the effect of glibenclamide (100 μM; G) alone or combined with 4, 4'-diisothiocyanostilbene 2, 2'-disulfonic acid (DIDS) (100 μM; G+D) or DIDS (100 μM; D) or combined with glibenclamide (100 μM; D+G) on (a) PAR2 synthetic ligand **[SLIGRL-NH_2]** (SEQ ID NO:2) (30 μM), (b) ATP (10 μM) and (c) PGE_2 (10 μM) in isolated mouse trachea.

The paragraph beginning at page 36, line 17 has been amended as follows:

Figure 34 is a graphical representation depicting increases in Isc to PAR2 and PAR1-activating peptides **[SLIGKV]** (SEQ ID NO:3) and **[SFLLRN]** (SEQ ID NO:1), respectively in human bronchial epithelium. The maximal response of the tissue, elicited by isobutylmethylxanthine is also shown.

The paragraph beginning at page 37, line 1 has been amended as follows:

Figure 36 is a graphical representation showing the effect of inhaled **[SLIGRL]** (SEQ ID NO:2) on LPS induced neutrophil recruitment in murine lungs.

The paragraph beginning at page 37, line 4 has been amended as follows:

Figure 37 is a graphical representation of **[digitised]** digitized original chart recordings showing the smooth muscle relaxing effects of PAR1 and PAR2 peptide activators, **[SLIGKV]** (SEQ ID NO:3) and **[SFLLRN]** (SEQ ID NO:1) respectively and the PAR activating enzymes, thrombin (PAR1 selective) and trypsin (PAR2 selective) in isolated ring segments of monkey small bronchi. Traces are characteristic of similar tissues taken from four separate animals (two pigtail macaques; two cynamologus). The experimental details are similar to those for the mouse isolated bronchi. Briefly, approximately 2 mm long rings of small bronchi were mounted on wire hooks. In each trace, half log concentrations are not depicted for clarity. In some cases

PGE3 and isoprenaline (iso) were added to (1) show that these tissues were responsive to PGE2 and (2) to obtain maximum tissue relaxation.

The paragraph beginning at page 39, line 1 has been amended as follows:

Abbreviations used herein are as follows:

Ach	Acetylcholine
ATP	Adenosine 5′-triphosphate
F_{max}/$KPSS_{max}$	Maximum force of contraction (grams)
FITC	Fluorescein-isothiocynate
HbO	Oxyhaemoglobin
IBMX	isobutyl methylxanthine
Indo	Indomethacin
KPSS	Potassium -containing physiological salt solution
L-NOARG	N^G-Nitro-L-arginine
NO	nitric oxide
PACAP	Pituitary adenylyl cyclase activating peptide
PAR	Protease activated Receptor
PAR2-AP	PAR2 Activating Peptide
SK	Ca^{2+}-activated K^+ channel
To	optimal tissue stretch to give maximum active force
TRAP	Thrombin Receptor-Activating Peptide, **[SFLLRN-NH_2]** (SEQ ID NO:1)
VIP	Vasoactive intestinal peptide

The paragraph beginning at page 40, line 5 has been amended as follows:

Acetylcholine chloride, bovine serum albumin, bradykinin triacetate, carbachol, cycloheximide, haemoglobin (bovine plasma), histamine dihydrochloride indomethacin, (-)-isoprenaline, N^G-nitro-I-arginine, substance P (acetate salt) and α-thrombin (bovine serum) were obtained from Sigma (MO, U.S.A.). Actinomycin D, apamin, aspirin, brefeldin A, carbaprostacyclin, isobutylmethyl xanthine (IBMX), prostaglandin ethanolamide, 9, 11-dideoxy-9α 11α-methanoepoxy-prostaglandin F2α (U46619), prazosin hydrochloride and nifedipine were from Sapphire Bioscience (N.S.W., Australia). Trypsin (bovine pancreas) was from Worthington Biochem (NJ, U.S.A.) and **[SLIGRL-NH_2]** (SEQ ID NO:2), **[SLIGKV-NH_2]** (SEQ ID NO:3) and **[SFLLRN-NH_2]** (SEQ ID NO:1) were obtained from Auspep (Vic, Australia).

The paragraph beginning at page 41, line 3 has been amended as follows:

The right and left main bronchi and their first order branches of specific pathogen-free (SPF) Balb/c mice (15-20 g; either sex), Hartley tricolour guinea-pigs (300-400 g; male) and Sprague-Dawley rats (200-350 g; either sex), all killed by either cervical dislocation or overdosed (i.p.) with sodium pentobarbitone, were cleared of surrounding connective tissue, nerves and blood vessels under a dissecting microscope and placed in cold, carbogenated (95% v/v O_2, 5% v/v CO_2) Krebs solution (Kemp and Cocks, 1997). Human airway preparations (0.5-1 mm in external diameter) were dissected from lungs of cancer patients undergoing thoracotomy at The Royal Melbourne Hospital, Melbourne, Australia. The epithelium was removed from some bronchi either by mechanical abrasion of the luminal surface (guinea-pig) or by brief, gentle intraluminal flushing of the airways with Krebs containing 0.1% v/v Triton-X100 (mouse and rat). In each case, removal of the epithelium was verified histologically in 8 μm formalin-fixed, paraffin sections stained with haemotoxylin and eosin. Ring segments (approximately 2mm long) of bronchi and bronchioles were mounted in Krebs (37°C) on stainless steel wires (40 μm) in dual channel (5 ml) Mulvany-Halpern myographs (JP Trading, Aarhus, Denmark) to record changes in isometric force (Kemp and Cocks, 1997). After equilibration at a passive force between 0.2 g and 0.3 g, tissues were contracted to their maximum levels of active force (F_{max}) with acetylcholine (30 μM), thoroughly washed with Krebs and allowed to return to baseline. Various drugs or their vehicles were then added and 30 min later all tissues were contracted to approximately 40% F_{max} with titrated concentrations of carbachol (10-500 nM). The L-type voltage-operated Ca^{2+} channel inhibitor, nifedipine (0.3 μM) was added to all mouse and rat tissues after obtaining F_{max} to control characteristic phasic contractile activity with carbachol.

When a stable level of active force to carbachol was obtained, tissues were exposed to cumulatively increasing concentrations of the PAR1 and PAR2-activating enzymes, thrombin (bovine serum, Sigma, MO, USA) and trypsin (bovine pancreas, 3x crystallized, Worthington Biochem, NJ, USA) respectively, and their synthetic tethered ligand peptide sequences, **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) and **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) (each >95% purity; Auspep, Parkville, Australia).

The paragraph beginning at page 42, line 9 has been amended as follows:

To assess the effect of enzyme-mediated receptor desensitization on responses to the synthetic peptides, mouse bronchi were allowed to recover to their initial level of active force to carbachol following cumulative concentration-responses curves to trypsin (0.001-0.3 U/ml) or thrombin (0.001-0.3 U/ml) but with enzymes still present in the myograph chamber. When the force again reached a steady level, they were tested for **[desensitisation]** desensitization with maximum concentrations of trypsin and thrombin (0.3 U/ml). If no response occurred the tissues were then exposed to cumulative concentrations of either **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) or **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) (0.1-30 μM).

The paragraph beginning at page 43, line 8 has been amended as follows:

Male Sprague-Dawley rates (8 weeks) were **[anaesthetised]** anaesthetized (xylazine 10 mg/kg, ketamine 100 mg/kg and 50 mg/kg each 30 min thereafter, i.p.) and cannulae were placed in the trachea, **[carotide]** carotid artery and jugular vein. Spontaneous breathing was stopped by an intravenous injection of pancuronium bromide (0.4 mg/kg and 0.2 mg/kg each 30 min thereafter) and rats were ventilated (tidal volume 8 ml/kg at 90 breaths/min, SAR-830 ventilator, CWE Inc., Ardmore, USA). Breath-to-breath measurement of airway resistance (R_L) and dynamic compliance (C_{dyn}) were calculated from flow and transpulmonary pressure recordings (PMS800, Mumed, London, UK). Flow was measured over the tracheal cannula (Fleisch pneumotachograph, Lausanne, Switzerland) and transpulmonary pressure was measured with a differential pressure transducer, one end being connected to the outlet of the tracheal cannula, the other to an air-filled cannula inserted in the esophagus. A rectal probe was issued to monitor body temperature. Serotonin (5-HT; 0.3 mg/kg i.v.) was administered as a bolus dose at 5 min intervals until reproducible changes in R_L and C_{dyn} were obtained. Prior to each 5-HT challenge, lungs were hyperinflated once (by delivering twice the tidal volume) to prevent and reverse atelecasis. **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2), the scrambled peptide **[LSIGRL-**

NH_2[<400>4]] (SEQ ID NO:4) (both 0.1 mg/ml) and their vehicle controls (saline) were then delivered for 30 sec as aerosols generated by an ultrasonic nebuliser (AeroSonic 5000, DeVilbiss, Somerset, USA) in series with a second ventilator and the response to 5-HT determined 5 min later.

The paragraph beginning at page 44, line 18 has been amended as follows:

Fresh frozen, paraformaldehyde-fixed sections (14 μm) of mouse bronchus were incubated with a rabbit antiserum directed against the carboxyl-terminal of mouse PAR2 **[(CSVKTSY[<400>5])]** (SEQ ID NO:5) at a dilution of 1:500 for 48 h, washed with phosphate-buffered saline (PBS) and then incubated with a biotinylated donkey anti-rabbit antiserum (Amersham) for 2 h, washed again with PBS and then labelled with FITC-conjugated streptavidin (Amersham) all at room temperature. After a final wash in PBS, the sections were mounted in buffered glycerol and viewed under a Biorad MRC1000 confocal scanning laser system installed on an Olympus IMT2 microscope with a krypton/argon laser. Visualisation of FITC was achieved using a 488nm excitation filter and a 522/535nm emission filter. Images of 768x612 pixels were then processed using Adobe Photoshop software. No staining was observed when the antiserum was preabsorbed with the immunizing peptide sequence (10 μM at 4°C for 24 h).

The paragraph beginning at page 46, line 24 has been amended as follows:

Mouse bronchus preparations were carefully mounted horizontally on fine (40 μm) stainless steel wires attached to the jaws of a Mulvany-Halpern myograph. After 60 min at 37° C, all rings were stretched to 0.5g passive force, which had been determined in preliminary experiments to be optimal, and allowed to recover from that stretch for a further 30 min. Maximum contraction (F_{max}) in each tissue was then determined with exogenously applied acetylcholine (ACh; 30μM) followed by washout. A further 30 min equilibration time was allowed before the tissues were actively contracted to between 20% and 60% of their individual F_{max} values with titrated concentrations of carbachol (10-100nM). When these contractions maintained steady plateaus, cumulative half-log concentrations or units of enzyme activity of trypsin, thrombin, **[SLIGRL-NH_2]** (SEQ ID NO:2) (PAR2-AP), **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP) prostaglandin E2 (PGE_2) and isoprenaline were added. In some cases, tissues were treated with a range of drugs prior to contraction to approximately 50% F_{max}. These included the cyclooxygenase inhibitors indomethacin and aspirin, the nitric oxide (NO) synthase

inhibitor N^G-nitro-L-(L-NOARG), the NO scavenger oxyhaemoglobin (HbO), and the L-voltage-operated Ca^{2+} channel inhibitor nifedipine.

The paragraph beginning at page 50, line 5 has been amended as follows:

Whenever attempts to remove the epithelium from mouse bronchi or trachea were made, they invariably damaged the underlying smooth muscle, since all rings treated in this manner failed to contract to ACh. Therefore, studies were conducted using the guinea-pig to obtain information as to the possible role of the epithelium in mediating bronchial smooth muscle relaxation to PARs. Concentration dependent relaxations to PAR2-AP were observed in six out of thirteen bronchial rings in which the epithelium was intact; the remaining seven tissues either gave no response or small contractions to PAR2-AP. In the same number of epithelium-denuded rings (n=6) from animals where PAR2-AP caused relaxation (n=6), PAR2-AP either caused a small contraction or no response, as seen in Figure 5. In a further experiment where spiral strips rather than rings were used, PAR2-AP caused relaxation which was clearly concentration- and epithelium-dependent, as shown in Figure 6. The presence and absence of the epithelium was histologically confirmed. **[SFLLRN-NH2]** (SEQ ID NO:1) (TRAP) only caused concentration-dependent contractions, which were unaffected by removal of the epithelium.

The paragraph beginning at page 51, line 4 has been amended as follows:

Figure 7 shows how isolated mouse bronchi were set up to measure relaxation sensitively. After an initial passive stretch to 0.5g (Ti) and recovery, each ring was contracted with acetylcholine (Ach; 30 μM). The contraction was taken as the tissue maximum and referred to as F_{max}. After washout (w) and recovery, the tissue was then contracted to approximately 40% F_{max} with titrated, cumulative concentrations of carbachol, resulting in a change in gain. When the contraction to carbachol reached a stable plateau, cumulative, half-log molar concentrations of PAR2-AP and TRAP were added. The results demonstrate that both PAR2-AP and **[SFLLRN-NH2]** (SEQ ID NO:1) (TRAP) caused powerful concentration-dependent relaxations in this preparation. These responses were unaffected by the combined treatment with the NO blockers L-NOARG (100 μM) and HbO (20 μM), but were abolished by the cyclooxygenase inhibitors indomethacin (3 μM) and as pirin (100 μM), as shown in Figure 8. TRAP was less effective as a mediator of relaxation, and responses to this ligand were converted to concentration-dependent contractions by indomethacin and aspirin. This effect was partially blocked by L-NOARG and HbO, as seen in Figure 8. Under the same bioassay conditions,

trypsin cause activity-dependent relaxation which, as for PAR2-AP, was also blocked by indomethacin. By contrast, thrombin caused only poor indomethacin sensitive relaxation at high concentrations which, like TRAP, were converted to contractions by indomethacin. These results are shown in Figure 8. Continual exposure of the mouse bronchi to high cumulatively increasing concentrations of PAR2-AP (up to 100 μM) for 2h allowed by washout had no effect on the sensitivity or maximum response to subsequent addition of PAR2-AP. All occurrences of relaxation were due to an indomethacin- and aspirin-sensitive mechanism, with no role for NO. Indomethacin and aspirin also converted the relaxation in response to **[SFLLRN-NHz]** (SEQ ID NO:1) (TRAP) to a contraction. Thrombin gave little or no relaxation in the absence of indomethacin, but like TRAP caused a contraction in its presence. In contrast, the response to PAR2-AP was virtually abolished after continual exposure of the tissue to a maximum concentration of trypsin, but not thrombin, as shown in Figure 9, indicating that trypsin and PAR2-AP activated the same receptor. PGE_2 caused potent and maximum relaxation of the mouse bronchi, as shown in Figure 10.

The paragraph beginning at page 54, line 7 has been amended as follows:

Bronchi from male Sprague-Dawley rats prepared as rings in the same way as for mouse bronchi produced results that were qualitatively similar to those observed in the mouse, as indicated in Figure 15. The PAR1 activating peptide **[SFLLRN-NHz]** (SEQ ID NO:1) (TRAP) only caused a contraction, whereas thrombin caused a small relaxation.

The paragraph beginning at page 56, line 7 has been amended as follows:

This tissue relaxed in an apparently apaminin sensitive manner to **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP) and to a lesser extent PAR2-AP. Thrombin, however, did not result in relaxation in this tissue, as depicted in Figure 18.

The paragraph beginning at page 57, line 18 has been amended as follows:

Aortic ring segments were contracted to approximately 50% $KPSS_{max}$ with titrated concentrations of the thromboxane A_2 mimetic, U46619 (1 to 10 nM). Once the U46619-induced contraction had reached a stable level, cumulative concentration-response curves to thrombin and trypsin (0.0001 to 1 U/ml, or the human PAR1 activating peptide **[(SFLLRN-NH_2)]** (SEQ ID NO:1), the human PAR2 activating peptide **[(SLIGKV-NH_2)]** (SEQ ID NO:3) or the mouse PAR2 activating peptide **[(SLIGRLN-H_2)]** (SEQ ID NO:2) (0.01 to 100 μM) were generated in the presence of bovine serum albumin (BSA; 0.005%). At the completion of each

curve, maximum endothelium-dependent and -independent relaxation for each ring segment was determined with the addition of substance P(2 nM) and isoprenaline (1 μM), respectively.

The paragraph beginning at page 58, line 12 has been amended as follows:

Tissue were either left untreated or treated with cumulative additions of one of thrombin (0.1 U/ml) or trypsin (0.1 U/ml) every 30 min for 2 h in the presence of BSA (0.005% w/v). Tissues were then washed thoroughly with Krebs solution and contracted to approximately 50% $KPSS_{max}$ with U46619. Tissues were then exposed to the enzyme (0.1 U/ml) with which they had previously been treated until no further relaxation was observed. Importantly, the tissues were washed with Krebs solution, containing an appropriate concentration of U46619 to maintain the precontraction, between treatments with each activating enzyme. This ensured that receptor desensitization was not masked by occupation of the receptor by the tethered ligand sequence. Once desensitization was achieved, cross-desensitization was investigated by addition of the enzyme (0.1 U/ml) not used in the desensitization process. Following this, cumulative concentration-response curves to the mouse PAR1 activating peptide, **[SLIGRL-NH_2]** (SEQ ID NO:2), were generated. Again, substance P(3 nM) and isoprenaline (1 μM) were then added to determine maximal endothelium-dependent and -independent relaxations, respectively.

The paragraph beginning at page 60, line 1 has been amended as follows:

The PAR1 activating peptide, **[SFLLRN-NH2]** (SEQ ID NO:1), also caused potent relaxation of precontracted human coronary artery segments, with pEC_{50} (-log M) and R_{max} values of 6.9 ± 0.1 and 95.2 ± 1.3% (n=10, from five patients), respectively. This relaxation was abolished by endothelium denudation, as shown in Figure 21. In contrast, responses to the human PAR2 activating peptide **[SLIGKV-NH_2]** (SEQ ID NO:3) were significantly less (R_{max} 39.9 ± 11.0%; n=5, from two patients). Interestingly, the mouse PAR2 activating peptide, **[SLIGRL-NH2]** (SEQ ID NO:2), which has a similar sequence to the human PAR2 activating peptide and has been shown to be equally active on PAR2 in other preparations (Blackhart et al., 1996), caused no relaxation.

The paragraph beginning at page 60, line 13 has been amended as follows:

[Desensitisation] Desensitization of tissues with either thrombin or trypsin caused loss of responsiveness to maximum relaxation-inducing concentrations of both enzymes, as shown in Figures 22a and 22b, indicating that the receptor(s) involved are activated by either of these enzymes. Interestingly, under these desensitizing conditions, the maximum response to

[SFRLLN-NH$_2$] (SEQ ID NO:1) was unaffected (R_{max} 92.0 ± 5.0), although there was a small, but significant ($P<0.05$) decrease in sensitivity (pEC_{50} 7.0 ± 0.1 vs 6.4 ± 0.2; n=8, from 4 patients), as illustrated in Figure 23.

The paragraph beginning at page 61, line 11 has been amended as follows:

As has been demonstrated in the vasculature of the rat (Saifeddine et al., 1996) and pig (Hwa, et al., 1996), the present studies show that U46619-contracted human coronary artery ring preparations were induced to relax by both thrombin and trypsin. However, only the peptide fragment corresponding to the human PAR1 tethered ligand sequence **[(SFLLRN-NH$_2$)]** (SEQ ID NO:1) was fully active in this preparation, while the PAR2 tethered ligand sequence **[(SLIGKV-NH$_2$)]** (SEQ ID NO:3) induced only a partial reversal of the U466-19-induced contraction at comparably high concentrations.

The paragraph beginning at page 61, line 19 has been amended as follows:

Responses to both thrombin and trypsin were entirely dependent on the presence of an intact endothelium, and were virtually abolished by a combination of L-NOARG-mediated inhibition of endothelial NO production and scavenging of residual NO by HbO, indicating that PAR induced relaxations were mediated by endothelium-derived NO. The degree of inhibition was similar to that observed with bradykinin in this study, and is consistent with other reports that endothelium-dependent relaxation of human coronary vessels is mediated predominantly by NO for agents including bradykinin (Kemp & Cocks, 1997) and substance P (Chester et al., 1990). Others have also shown that PAR-mediated vasodilatation in rat (Muramatsu et al., 1992), pig (Tesfamarium et al., 1993) and dog (Tesfamarium, 1994) vessels is due to endothelial cell-derived NO. In contrast to previous reports which showed that thrombin contracted endothelium-denuded preparations of coronary artery from dog (White, 1994; Tesfamarium, 1994) and pig (Glusa & Markwardt, 1998), neither thrombin nor trypsin induced contraction of endothelium-denuded human artery preparations in the present study. The lack of contraction to thrombin may be explained by the observation that mRNA for PAR1 was present only in endothelial cells in normal, non-atherosclerotic arteries (Nelken et al., 1992). Whether thrombin or the PAR1 activating peptide can cause contraction of endothelium-free vessels obtained from patients suffering from atheroma is of interest, sine Nelken et al. (1992) also located PAR1 mRNA in smooth muscle cells in affected vessels. The observations suggest that both enzymes mediate relaxation by PAR1 activation. However, although trypsin can cleave and activate

PAR1, as shown in Vu et al. (1991) the concentrations required (≥ 25 U/ml or 50 nM) are far in excess of those observed in the present studies on human coronary arteries to cause endothelium-dependent relaxation (0.01 - 1 U/ml or 0.02-2 nM). The low potency of the human PAR2 tethered ligand sequence, **[SLIGKV-NH_2]** (SEQ ID NO:3), and the lack of activity of the equivalent murine sequence, **[SLIGRL-NH_2]** (SEQ ID NO:2) could initially be taken as evidence for the sole presence of PAR1 in human coronary arteries, with the specificity of this peptide being lost at high concentrations leading to "cross over" activation of PAR1. The human PAR2 tethered ligand sequence, **[SLIGKVD-NH_2]** (SEQ ID NO:7), however, does not activate PAR1 at concentrations up to 1 mM in human platelets (Blackhart et al., 1996) - far in excess of those used in this study. Furthermore, structure-activity studies have shown that PAR1 activating peptides lacking an aromatic residue at position 2 (as is the case with **[SLIGKV-NH_2]** (SEQ ID NO:3)) are incapable of activating PAR1 in both transfected cell lines (Nystedt et al., 1995) and human platelets (Scarborough et al, 1992; Vassallo et al., 1992). Therefore, PAR1, and to a lesser extent PAR2, may exist in human coronary endothelial cells. Such a conclusion, however, is contrary to the present findings that heterologous desensitization was induced by either thrombin or trypsin.

The paragraph beginning at page 63, line 12 has been amended as follows:

Such evidence suggests a single receptor type. In porcine coronary arteries, which are known to express both PAR1 and PAR2 (Hwa et al, 1996), heterologous desensitization was observed with high concentrations of trypsin, but only homologous desensitization occurred with thrombin (Hwa et al, 1996). Thus, while cross-desensitization and the poor sensitivity of **[SLIGKV-NH_2]** (SEQ ID NO:3) and **[SLIGRL-NH_2]** (SEQ ID NO:2) point to their involvement of a single receptor population, the ability of relatively low concentrations of trypsin to mediate relaxation similar to those observed with thrombin is inconsistent with the view that a 'typical' thrombin receptor is involved.

The paragraph beginning at page 64, line 15 has been amended as follows:

The "atypical" thrombin receptor in the human coronary artery endothelial cell appears to be sensitively activated by both thrombin and trypsin *via* either a common or dual enzyme binding site(s). Further support for the existence of such a receptor is provided by the observation that **[SLIGKV-NH_2]** (SEQ ID NO:3) is capable of inducing vasodilatation despite the lack of the critical aromatic residue at position 2. Therefore, without wishing to be bound by

any proposed theory, the inventors believe that the receptor responsible for endothelium-dependent relaxation of human coronary artery is a PAR1-like receptor, which has a modified amino-terminal exodomain comprising a trypsin binding domain and a modified tethered ligand binding region containing different pharmacophore **[specifcities]** specificities.

The paragraph beginning at page 65, line 1 has been amended as follows:

This study also shows that complete desensitization of responses to both thrombin and trypsin had only a small inhibitory effect on the responses of the arteries to **[SFLLRN-NH_2]** (SEQ ID NO:1), which is contrary to earlier reports using pig coronary artery (Tesfamarian, 1994; Hwa, 1996) and rat aorta (Hollelberg et al., 1996). However, differences in desensitization procedures between these previous studies and the present one might provide clues as to how PAR responsiveness is regulated following enzymic activation. In the present study, high concentrations of both thrombin and trypsin were used for 2 to 3 hours, followed by approximately 30 minutes recovery while enzyme washout and tissue contraction occurred. This resulted in complete loss of responsiveness to both thrombin and heterologous desensitization with trypsin were observed. However, in each case, responsiveness to **[SFLLRN-NH_2]** (SEQ ID NO:1) and **[SLIGKV-NH_2]** (SEQ ID NO:3) was maintained. In the study of Hwa et al (1996), a high enzyme concentration was used over a much shorter contact time (10 to 20 minutes), and importantly, the enzyme was not washed out. The results showed a loss of responses to **[SFLLRN-NH_2]** (SEQ ID NO:1) after homologous desensitization with thrombin, and loss of both **[SFLLRN-NH_2]** (SEQ ID NO:1) and **[SLIGKV-NH_2]** (SEQ ID NO:3) responses following heterologous desensitization with trypsin.

The paragraph beginning at page 65, line 21 has been amended as follows:

The retention and loss of responses to the tethered ligand sequences following desensitization shown in the present studies and in that of Hwa et al (1996) may reflect the rates of internalization and recycling of PARs following enzymic activation. Both PAR1 and PAR2 are rapidly internalized upon enzymic activation, stimulating the mobilization of a pool intact, pre-formed receptors which are rapidly (<30 minutes) inserted into the cell membrane (Bohm et al, 1996; Hein et al, 1994; Hoxie et al, 1993). The loss of subsequent enzyme-induced responses observed by Hwa et al (1996) using a rapid desensitization technique could be explained by the inability of the cell to replenish cell surface receptors from its intracellular reserve over this short period. With the prolonged desensitization technique used in this study, any reserves of

intracellular receptors would probably have been depleted. Despite this, responses to **[SFLLRN-NH_2]** (SEQ ID NO:1) were only minimally affected.

The paragraph beginning at page 67, line 10 has been amended as follows:

Like the mouse tissues, the human bronchioles were contracted to approximately 30-40% F_{max} with titrated cumulative concentrations of carbachol. Figures 24 and 25 show that both thrombin and trypsin caused activity dependent relaxation that was reversed by indomethacin. In contrast, only the PAR1-activating peptide **[SFLLRN-NH_2]** (SEQ ID NO:1) (TRAP) relaxed the bronchioles. The PAR2-activating peptide, **[SLIGRL-NH_2]** (SEQ ID NO:2) (PAR2-AP) appeared to cause some contraction, but this was most likely baseline drift.

The paragraph beginning at page 68, line 12 has been amended as follows:

Fresh frozen, paraformaldehyde-fixed sections (14 μM) of mouse bronchus ere incubated with a rabbit antiserum directed against the carboxy-terminal of mouse PAR2 **[(CSVKTSY)]** (SEQ ID NO:5) at a dilution of 1:500 for 48 h, washed with PBS and then incubated with a biotinylated donkey anti-rabbit antiserum (Amersham) for 2 h, washed against with PBS, and then labelled with FITC-conjugated streptavidin (Amersham), all at room temperature. After a final wash in PBS, the sections were mounted in buffered glycerol and viewed under a Biorad MRC1000 confocal scanning laser system installed on an Olympus IMT2 microscope with a krypton/argon laser. **[Visualisation]** Visualization of FITC was achieved using a 488 nm excitation filter and a 522/535 nm emission filter. Images of 768 x 612 pixels were then processed using Adobe Photoshop software.

The paragraph beginning at page 69, line 15 has been amended as follows:

Using an antibody directed against the carboxyl terminal of mouse PAR2 and confocal fluorescence microscopy, the inventors found specific PAR2 immunoreactivity localized to epithelial cells, often focally within the cytoplasm, as well as to smooth muscle cells and fibroblasts in the submucosa of the mouse bronchus (Figure 26). In functional studies, the mouse PAR2 tethered ligand sequence, **[SLIGRL-NH_2([<400>2]]** (SEQ ID NO:2); Nystedt et al, 1994) and trypsin each caused concentration-dependent, rapid onset and near-maximum relaxation of mouse bronchial rings contracted with the stable muscarinic agonist carbachol. These relaxations were abolished by either removal of the epithelium or inhibition of cyclooxygenase (Figures 1 and 26). For **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) the sensitivity (pEC_{50}, -log M) was 5.6 ± 0.1 and the maximum relaxation (R_{max}) was 94 ± 3%. Similar concentration-dependent

relaxations were obtained from the PAR1 tethered ligand sequence **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1); Déry et al, 1988; λpEC_{50}, 5.6 ± 0.1; R_{max}, 76 ± 11%) and thrombin. In contrast to PAR2 activation, removal of the epithelium or inhibition of cyclooxygenase unmasked smooth muscle contractions to PAR1 activation with **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) (Figures 1 and 26). Unlike **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) (Blackhart et al, 1996) which is a specific activator of PAR2, **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) can activate both PAR1 and PAR2. However, the inability of **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) to contract epithelium-denuded or cyclooxygenase-blocked preparations of the mouse bronchi indicates that **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) causes smooth muscle contraction *via* activation of PAR1. It is clear that the relaxations observed in response to **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) or low concentrations of trypsin were due to activation of epithelial PAR2 or an unidentified receptor with similar sensitivity to **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) and trypsin. This is confirmed by the one observation that the responses to **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) were abolished by prior desensitization to trypsin but were unaffected by thrombin desensitization whilst those to **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) were abolished following desensitization to both thrombin and trypsin.

The paragraph beginning at page 70, line 19 has been amended as follows:

Relaxations to **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) and **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) in the mouse bronchi were not due to nitric oxide (NO) since they were unaffected by the NO synthase inhibitor, N^G-nitro-L-arginine (100 μM) and the NO scavenger, oxyhaemoglobin (20 μM; Figure 26). Therefore, a prostanoid rather than NO mediated the relaxations of both PARs. PGE_2 is a likely candidate, since it is the most prevalent prostanoid released from the airway epithelium and the inventors found it to sensitively and powerfully relax mouse bronchi (pEC_{50}, 8.2 ± 0.1; R_{max}, 100%, Figure 1).

The paragraph beginning at page 71, line 3 has been amended as follows:

Smaller, intrapulmonary airways are likely to contribute more than larger airways to resistance to flow in the lungs. Therefore, the inventors investigated the effects of PAR-activating peptides in first generation branches of the mouse main bronchi. The inventors observed similar indomethacin-sensitive relaxations to the PAR ligands in these preparations although the sensitivity and maximum relaxation to both **[SFLLRN-NH_2[<400>1]]** (SEQ ID NO:1) (pEC_{50}, 5.5 ± 0.02; R_{max}, 58 ± 10%) and **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2)

(pEC_{50}, 5.1 ± 0.05; R_{max}, 58 ± 4%) were significantly less ($P<0.05$) than those in the main bronchi (Figure 26).

The paragraph beginning at page 72, line 10 has been amended as follows:

In addition to the mouse, the inventors also observed PAR-mediated bronchorelaxation in the airways of other species. Thus, **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) caused epithelium-dependent and indomethacin-sensitive relaxations in rat isolated bronchi (pEC_{50}, 5.5 ± 0.1; R_{max} 56 ± 5%) and bronchioles (pEC_{50}, 5.1 ± 0.1; R_{max} 67 ± 5%) and similar potency (pEC_{50}, 5.4 ± 0.2), epithelium-dependent relaxation in the guinea-pig isolated bronchi but with a significantly ($P<0.05$) lower R_{max} (31±5%) than those in both rat and mouse bronchi. Also, from experiments (n=4), the inventors observed PAR2-mediated relaxations in human intrapulmonary airways which, although weak by comparison with those in mice, were blocked by indomethacin.

The paragraph beginning at page 72, line 21 has been amended as follows:

Importantly, the inventors have demonstrated here that **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) is a highly effective inhibitor of bronchoconstriction *in vivo*. Thus, a 30 sec exposure to an aerosol of a0.1 mg/ml solution of **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2), but not the scrambled peptide sequence **[LSIGRL-NH_2[<400>4]]** (SEQ ID NO:4) caused inhibition (50-70%) of 5-hydroxytryptamine (5-HT)-induced changes in airway resistance (R_L) and dynamic compliance (C_{dyn}) in an anaesthetized rats (Figure 11b). This effect **[SLIGRL-NH_2[<400>2]]** (SEQ ID NO:2) could be functionally antagonized by higher doses of 5-HT.

The paragraph beginning at page 75, line 7 has been amended as follows:

In addition to the findings in mouse bronchi, shown in the earlier Examples, indomethacin-sensitive relaxations to both the PAR1- and PAR2-activating peptides were also observed in first branches of the main bronchi of the mouse, which the inventors have termed bronchioles. However, the sensitivity and maximum relaxation to **[SFLLRN-NH_2]** (SEQ ID NO:1) (pEC_{50} 5.5 ± 0.2; R_{max}, 58 ± 10%) and **[SLIGRL-NH_2]** (SEQ ID NO:2) (pEC_{50} 5.1 ± 0.05; R_{max}, 58 ± 4%) were significantly less ($P<0.05$) than those shown in Figure 27 for the main bronchi. Similar responses to **[SLIGRL-NH_2]** (SEQ ID NO:2) were observed in other species. Thus, **[SLIGRL-NH_2]** (SEQ ID NO:2) caused indomethacin-sensitive relaxations in rat bronchi (pEC_{50} 5.5 ± 0.1; R_{max}, 56 ± 5%) and bronchioles (pEC_{50} 5.1 ± 0.1; R_{max}, 67 ± 5%) and similar potency (pEC_{50} 5.4 ± 0.2%) epithelium-dependent relaxation in the guinea-pig but with significantly ($P<0.05$) lower efficacy (R_{max} 31 ± 5%) than in both rat and mouse bronchi.

Furthermore, in preliminary experiments, the inventors observed PAR2 mediated relaxation in human bronchi (n=4), which in one case was blocked by indomethacin. The similar potencies for **[SLIGRL-NH$_2$]** (SEQ ID NO:2) in mice, rats and guinea-pigs indicate expression of a similar receptor, whilst the different efficacies suggest either different receptor numbers or coupling between species. The rank order of efficacies for **[SLIGRL-NH$_2$]** (SEQ ID NO:2), mouse > rat > guinea-pig, however, contrasts with the severity of symptoms in allergic models of asthma. For example, mice show resistance to immunological challenge including only a small degree of airway hyperreactivity (AR) compared with rats and guinea pigs, the latter of which show characteristic high levels of $A4^{16}$ and may die when exposed to similar immunological challenges. One reason why mice appear relatively asymptomatic when used in immunological models of asthma may in part be due to a higher relative effectiveness of their PAR2-dependent bronchoprotective mechanism.

The paragraph beginning at page 76, line 7 has been amended as follows:

The mouse PAR2 tethered ligand sequence, **[SLIGRL-NH$_2$]** (SEQ ID NO:2) (Nystedt et al, 1994) and trypsin each caused concentration-, epithelium- and cyclooxygenase-dependent, rapid onset and near-maximum relaxations of mouse bronchial rings contracted with the stable muscarinic agonist carbachol, as shown in Figures 26b and c and Figure 27. For **[SLIGRL-NH$_2$]** (SEQ ID NO:2) the sensitivity (pEC_{50}, -log M) was 5.6 ± 0.1 and the maximum relaxation (R_{max}) was 94 ± 3%. Similar concentration-dependent relaxations were also obtained to the PAR1 tethered ligand sequence **[SFLLRN-NH$_2$]** (SEQ ID NO:1) (Dery et al., 1998) [pEC_{50} 5.6 ± 0.1; R_{max} 76 ± 11%] and thrombin. In contrast, PAR2, however, both removal of the epithelium and inhibition of cyclooxygenase with either indomethacin or aspirin unmasked direct smooth muscle contractions to PAR1 activation, as shown in Figures 26 and 27. Neither of the relaxations to **[SLIGRL-NH$_2$]** (SEQ ID NO:2) and **[SFLLRN-NH$_2$]** (SEQ ID NO:1) was due to nitric oxide (NO11, since they were completely unaffected by the NO synthase inhibitor, N^G-nitro-L-arginine (100 μM) either alone, or in combination with the NO scavenger, oxyhaemoglobin (20 μM), a combination of NO inhibitors which abolishes all NO release from vascular endothelial cells *in situ* (Drummond and Cocks, 1996; Kemp and Cocks, 1997). Therefore, these results indicate that a prostanoid released from the epithelium mediated the relaxations to both PARs. PGE_2 is a likely candidate, since it is the most prevalent prostanoid released from the airway epithelium, as

shown in Figure 26, and the inventors found it to be a potent bronchodilator in this tissue, causing 100% relaxation with a pEC_{50} of 8.2 ± 0.1 (n=6).

The paragraph beginning at page 77, line 6 has been amended as follows:

The relaxations to **[SLIGRL-NH_2]** (SEQ ID NO:2) and **[SFLLRN-NH_2]** (SEQ ID NO:1) in the mouse bronchi were likely to have been due to activation of separate receptors, since those to **[SLIGRL-NH_2]** (SEQ ID NO:2) were abolished by prior desensitization to trypsin but not thrombin whilst those to **[SFLLRN-NH_2]** (SEQ ID NO:1) were inhibited by both thrombin and trypsin, as shown in Figure 28. Also, desensitization with **[SLIGRL-NH_2]** (SEQ ID NO:2) blocked the response to trypsin. This pattern of activity agrees with previous reports showing that thrombin only activates PAR1 while higher concentrations of trypsin can activate both PAR2 and PAR1 (Vu et al., 1991; Molino et al, 1997).

The paragraph beginning at page 77, line 16 has been amended as follows:

Mice were challenged with bacterial lipopolysaccharide (LPS; 10 μg/mouse) via intranasal administration under light halothane anesthesia. Prior to this challenge, mice were treated with **[SLIGRL-NH2]** (SEQ ID NO:2) (2 mg/kg or 20 mg/kg) or saline (control) via the same route of administration.

The paragraph beginning at page 77, line 21 has been amended as follows:

Three hours after receiving LPS, the mice were killed via an interperitoneal injection of sodium pentabarbitone and the lungs were cannulated and lavaged with 4 × 0.5 ml washes of phosphate buffered saline. The total number of cells retrieved by this procedure was determined with a haemocytometer. Differential counts of individual cell types were performed on cytospin preparations of the lavage fluid, stained with the conventional May-Grünwald/Giemsa blood stain. The total number of neutrophils in each sample was then calculated from the proportion of neutrophils in the cytospin preparations, as a proportion of the total number of cells retrieved. In animals which received neither LPS or **[SLIGRL-NH2]** (SEQ ID NO:2), very few neutrophils were observed.

The paragraph beginning at page 78, line 7 has been amended as follows:

Figure 36 shows group data for n=7 controls, n=3 at 2 mg/kg **[SLIGRL]** (SEQ ID NO:2) and n=6 at 20 mg/kg **[SLIGRL]** (SEQ ID NO:2). Clearly prior treatment with **[SLIGRL]** (SEQ ID NO:2) causes a dose-dependent inhibition of the increase of neutrophil infiltration into the lungs in response to LPS.

The paragraph beginning at page 79, line 10 has been amended as follows:

After an equilibration time of 30 min, compounds for testing and drugs to elucidate the mechanism of any change sin Isc to the test compounds were added to either side of the tracheal epithelium (i.e., to either bath). Compounds used for increasing Isc were PGE_2, adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP) and the PAR1-, PAR-2 and PAR-4 activating peptides, **[SFLLRN]** (SEQ ID NO:1) and **[SLIGRL]** (SEQ ID NO:2) and **[GYPGQY]** (SEQ ID NO:8), respectively. These stimulants were added cumulatively with controls for any time-dependent tachyphylaxis.

The paragraph beginning at page 79, line 21 has been amended as follows:

Figure 30 shows typical original traces of the increases in Isc in response to luminally-applied AT, **[SLIGRL]** (SEQ ID NO:2), and PGE2. For all three cases, each concentration of activator caused a rapid initial rise in Isc usually followed by some degree of recovery until a steady plateau was reached. The group data for these and other activators is depicted in Figure 31. Figure 33 provides digitized traces of original chart recordings showing the effects of inhibitors of two distinct chloride channels on changes in Isc to single, submaximal concentrations of ATP, PGE2 and **[SLIGRL]** (SEQ ID NO:2). The drugs used were glibenclamide (G), an inhibitor of the cystic fibrosis transmembrane regulator (CFTR; Schultz et al., 1999) and DIDS, an inhibitor of calcium-activated chloride channels (CICa, Gruber, et al., 1999). From the group data for these experiments shown in Figure 33, it can readily be seen that whilst most of the chloride conductance to **[SLIGRL]** (SEQ ID NO:2) was due to the CFTR channel, a substantial amount remained due to CICa. These increases in Isc to **[SLIGRL]** (SEQ ID NO:2), ATP and PGE2 in the mouse airways were unaffected by the cyclooxygenase inhibitor, indomethacin.

The paragraph beginning at page 80, line 14 has been amended as follows:

Figure 34 depicts increases in Isc to PAR2- and PAR1-activating peptides, **[SLIGKV]** (SEQ ID NO:3) and **[SFLLRN]** (SEQ ID NO:1) respectively in the epithelium of a section of human intralobular bronchus. Prior to addition of the drugs shown, the preparation was incubated with amiloride, because secretory responses cannot be observed in human tissues unless sodium channels are inhibited by this drug. The maximum response elicited by the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX) is included. Note that the relatively poor response to the human PAR2-activating peptide, **[SLIGKV]** (SEQ ID NO:3), is

in keeping with other data presented herein for PAR2 function (epithelium-dependent smooth muscle relaxation) and the apparent low level of expression of the receptor as determined by immunohistochemistry.

The paragraph beginning at page 82, line 12 has been amended as follows:

Figure 37 is a representation of **[digitised]** digitized original chart recordings showing the smooth muscle relaxing effects of PAR1 and PAR2 peptide activators, **[SLIGKV]** (SEQ ID NO:3) and **[SFLLRN]** (SEQ ID NO:1) respectively and the PAR activating enzymes, thrombin (PAR1 selective) and trypsin (PAR2 selective) in isolated ring segments of monkey small bronchi. Traces are characteristic of similar tissues taken from four separate animals (two pigtail macaques, two cynamologus). The experimental details are similar to those for the mouse isolated bronchi. Briefly, approximately 2 mm long rings of small bronchi were mounted on wire hooks. In each trace, half log concentrations are not depicted for clarity. In some cases, PGE_2 and isoprenaline (iso) were added to (1) show that these tissues were responsive to PGE_2 and (2) to obtain maximum tissue relaxation.

The paragraph beginning at page 83, line 4 has been amended as follows:

Figure 38 provides cumulative concentration-response curves to the PAR1, PAR2 and PAR4-activity peptides, **[SFLLRN]** (SEQ ID NO:1), **[SLIGRL]** (SEQ ID NO:2) and **[GYPGKF]** (SEQ ID NO:6) and the PAR-activating enzymes, thrombin and typsin in isolated rat trachea (a, d) bronchi (b, e) and first bronchi (c, f). Values are expressed as percentage relaxation or contraction from carbachol-induced contraction (mean ± s.e. mean %, n=6-12). Confocal microscopic imaging was used to confirm the immunohistochemical localization of PAR2 in rat trachea, bronchi and intrapulmonary bronchi. Co-localization of PAR2 immunofluorescence with two separate PAR2 antibodies, PAR2-C antibody (green) and PAR2-N antibody (red) superimposed images show as yellow staining.

The paragraph beginning at page 84, line 8 has been amended as follows:

The studies described herein have identified functional PAR1 and PAR2 in the bronchi of mouse, rat, domestic pigs and guinea-pigs which, when activated by specific proteases, thrombin and trypsin or the human PAR1 and mouse PAR2 tethered ligand sequences, **[SFLLRN-NH_2]** (SEQ ID NO:1) and **[SLIGRL-NH_2]** (SEQ ID NO:2) respectively, cause profound relaxation of bronchial muscle. PAR2, and most likely PAR1 are located in the epithelium, and when activated, mediate smooth muscle relaxation via the release of endogenous prostaglandin (PG),

most likely PGE_2. This relaxation was as rapid and complete as that for isoprenaline, the clinically most efficacious and rapidly acting beta-adrenoreceptor agonist bronchodilator currently available.

A redline copy of the figures that have been amended is submitted herewith. Sheets 1, 25, 28, 29, 30, 31, 32, 34, 35, 36, 37, 38, 39, and 40 of the figures have been amended.

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